

Lipoprotein Release by Bacteria: Potential Factor in Bacterial Pathogenesis

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Lipoprotein (LP) is a major component of the outer membrane of bacteria in the family *Enterobacteriaceae*. LP induces proinflammatory cytokine production in macrophages and lethal shock in LPS-responsive and -nonresponsive mice. In this study, the release of LP from growing bacteria was investigated by immuno-dot blot analysis. An immuno-dot blot assay that could detect LP at levels as low as 100 ng/ml was developed. By using this assay, significant levels of LP were detected in culture supernatants of growing *Escherichia coli* cells. During mid-logarithmic growth, approximately 1 to 1.5 µg of LP per ml was detected in culture supernatants from *E. coli*. In contrast, these culture supernatants contained 5 to 6 µg/ml of lipopolysaccharide (LPS). LP release was not unique to *E. coli*. *Salmonella typhimurium*, *Yersinia enterocolitica*, and two pathogenic *E. coli* strains also released LP during in vitro growth. Treatment of bacteria with the antibiotic ceftazidime significantly enhanced LP release. Culture supernatants from 5-h cultures of *E. coli* were shown to induce in vitro production of interleukin-6 (IL-6) by macrophages obtained from LPS-nonresponsive C3H/HeJ mice. In contrast, culture supernatants from an *E. coli* LP-deletion mutant were significantly less efficient at inducing IL-6 production in C3H/HeJ macrophages. These results suggest, for the first time, that LP is released from growing bacteria and that this released LP may play an important role in the induction of cytokine production and pathologic changes associated with gram-negative bacterial infections.

Lipopolysaccharide (LPS), or endotoxin, is an integral component of the outer membrane of all gram-negative bacteria. The biologically active lipid A moiety of LPS is believed to play a central pathogenic role in sepsis and septic shock due to gram-negative bacteria (34). LPS is released by different bacteria during both in vitro and in vivo growth, and this release is significantly enhanced when the bacteria are lysed following exposure to antibiotics or human serum (2, 15, 16, 29, 32, 42, 47, 48). LPS released from the microbial surface is also believed to be more biologically active than microbe-associated LPS (28). The in vivo release of LPS has been proposed to be an important mechanism for inducing septic shock (1, 3, 25, 43). Circulating LPS has been detected and implicated in a variety of septic states and, when injected into animals, can evoke pathophysiologic responses that resembles gram-negative-bacterium-induced septic shock (18, 30). LPS does not injure host tissues directly but, rather, through the actions of a variety of inflammatory mediators induced by LPS exposure (13).

Although LPS has been clearly documented to play a potentially important role in septic shock induced by gram-negative bacteria, very little is currently known about the function of other bacterially derived components in septic shock and/or in the induction of cytokine production. In fact, there is significant evidence that other components of gram-negative bacteria also play an important role in the pathology associated with infections mediated by these organisms (11, 17, 26, 44). Although many studies have addressed different aspects of LPS release from bacteria, little is currently known about the fate of other outer membrane components during bacterial growth. We have recently shown that bacterial lipoprotein (LP) is important in the induction and pathogenesis of septic shock. LP

was shown to induce in vitro production of tumor necrosis factor alpha and interleukin-6 (IL-6) by mouse and human macrophages (51, 52) and to induce lethal shock and in vivo production of TNF- α and IL-6 in LPS-responsive and nonresponsive mice (53). More importantly, LP was shown to act synergistically with LPS to induce lethal shock and proinflammatory cytokine production, which suggests that LP and LPS activate cells via different mechanisms (53). LP is one of the most abundant proteins in the outer membranes of gram-negative bacteria of the family *Enterobacteriaceae* (10, 45). The possibility that LP, like LPS, is released by growing bacteria and/or lysed bacteria has never been investigated. Since LP can induce proinflammatory cytokine production, induce lethal shock, and act synergistically with LPS, the possibility that LP is released by growing and/or lysed bacteria becomes an important question for furthering our understanding of its role in the pathogenesis of gram-negative bacterial infections. In this report, we show that LP is released by growing bacteria and that this release is significantly enhanced when bacteria are exposed to the antibiotic ceftazidime. Additionally, we show that bacterial culture supernatants containing LP can induce IL-6 production in macrophages obtained from LPS-nonresponsive mice. These results suggest, for the first time, that LP, like LPS, can be released by growing or lysed bacteria and that this released LP may play an important role in the pathogenesis associated with gram-negative bacterial infections.

MATERIALS AND METHODS

Mice. C3H/HeJ mice were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). The mice were housed under specific-pathogen-free conditions. Female mice at 8 weeks of age were used in all experiments.

Bacteria. *Escherichia coli* K-12 and an *E. coli* K-12 strain that is an LP deletion mutant were obtained from Barbara Bachmann, *E. coli* Genetic Stock Center (New Haven, Conn.). The *E. coli* LP deletion mutant (JE 5505) was previously characterized (23, 51). *Yersinia enterocolitica* WA (O:8), *E. coli* 51, *E. coli* 331, *Salmonella typhimurium* TML, *Shigella flexneri* SA100, *Vibrio cholerae*, and *Pseudomonas aeruginosa* were provided by Robert Brubaker, Department of Microbiology, Michigan State University, East Lansing, Mich., or obtained from sources reported previously (22, 38).

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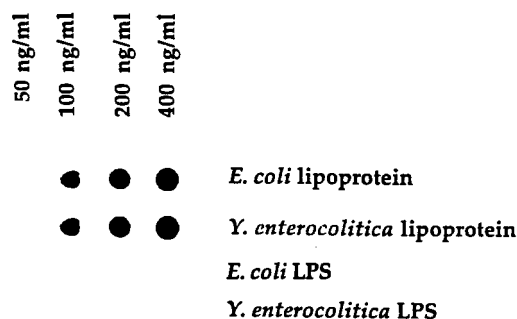


FIG. 1. Detection of LP by immuno-dot blot analysis. Purified *E. coli* LP and LPS in BHI broth were loaded onto nitrocellulose membranes and processed as described in Materials and Methods. Data are from one experiment that was representative of three separate experiments.

Bacterial cultures. Bacteria were inoculated into brain heart infusion (BHI) broth (2 ml) and incubated for 16 to 18 h at 37°C with aeration. The overnight culture was used to inoculate 100 ml of fresh BHI broth, which was incubated at 37°C with vigorous shaking. At various time points postinoculation, cultures were either assessed for bacteria (CFU) or sampled for the amount of LP versus LPS present in the culture supernatants. To assess LP versus LPS release from bacteria, 5 ml of bacterial culture was removed and centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatant was collected and assayed for LP and/or LPS content as described below. The bacterial pellet was reconstituted in 1 ml of 6 M guanidine HCl, sonicated for 5 min, and boiled for 10 min. The total volume was brought to 5 ml with phosphate-buffered saline (PBS) and then assessed for LP

versus LPS content. To determine bacterial growth at various time points, 1 ml of bacterial culture was removed and 10-fold dilutions were made with PBS. From each dilution, 100 μ l was plated onto L-agar plates, and colony counts were determined after overnight incubation at 37°C.

LP and LPS purification. LP and LPS were purified from *E. coli* K-12 as previously described (51, 53). LPS was purified from bacteria by hot-phenol extraction. LPS contained no detectable protein, either by silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gels or by the Coomassie blue binding assay. LP contained <25 pg of LPS/mg of protein.

Immuno-dot blot analysis. Various dilutions of purified *E. coli* LP or bacterial culture supernatants diluted in PBS were loaded (200 μ l per well) onto a nitrocellulose membrane under vacuum. The nitrocellulose membranes were blocked at 4°C overnight with 3% gelatin in 20 mM Tris buffer (pH 7.5) containing 0.5 M NaCl and 0.05% Tween 20 (TBST). The membranes were then incubated with a mouse monoclonal antibody (4C4) specific for LP (19) at 1 μ g/ml in TBST for 2 h at room temperature. After the membranes were washed (with TBST) six times, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad) was added at a dilution of 1/3,000 in TBST and the mixture was incubated for 1 h at room temperature. Finally, the membranes were washed as described above and developed in an enhanced chemiluminescent substrate (Pierce, Rockford, Ill.) for 5 to 10 min at room temperature before being exposed to medical X-ray film (Fuji Medical Systems U.S.A., Inc., Stamford, Conn.). The LP content of bacterial preparations and supernatants was estimated by comparing the dot blot results with results obtained from serial dilutions of purified LP that was used as a standard in the dot blot assay for comparison.

LPS determination. LPS was measured by the QCL-1000 *Limulus* amoebocyte lysate (LAL) assay (BioWhittaker, Inc., Walkersville, Md.) as specified by the manufacturer. Briefly, 50 μ l of the test sample was added in duplicate to 50 μ l of LAL in a pyrogen-free microtiter plate. The mixture was incubated at 37°C for 10 min, and 100 μ l of chromogenic substrate solution was added. After incubation at 37°C for 4 min, color development was terminated by addition of 100 μ l of 20% acetic acid. The optical density was measured at 405 nm, and the LPS

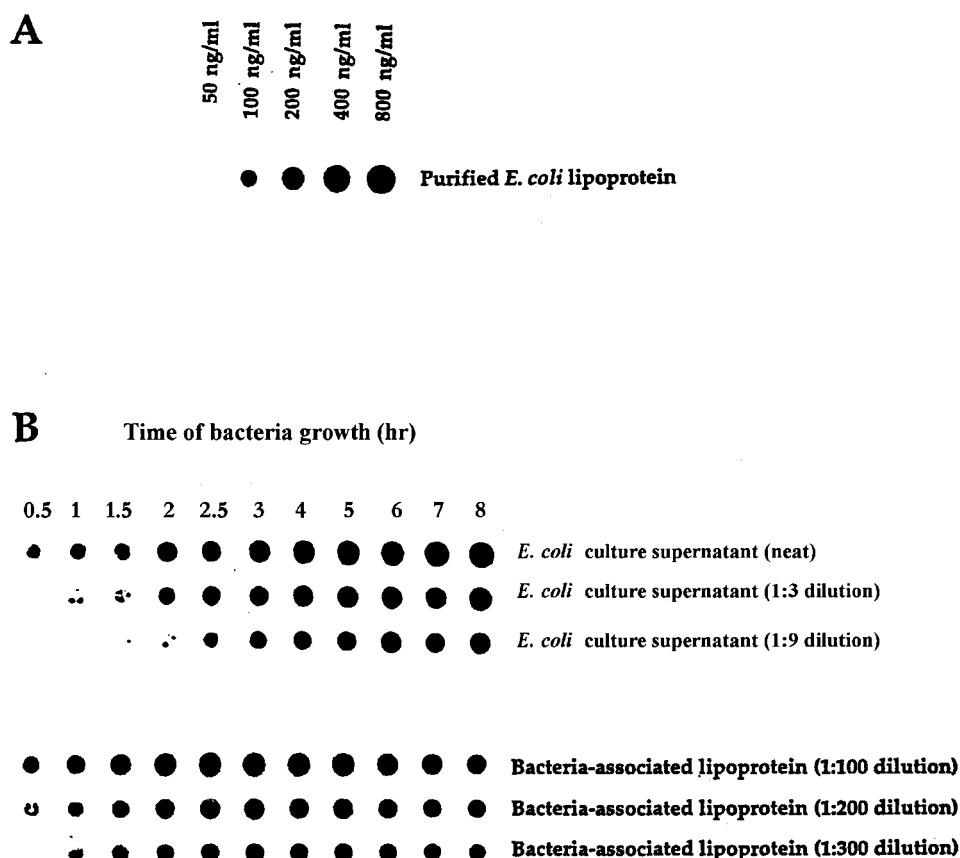


FIG. 2. LP can be detected in culture supernatants obtained from growing *E. coli*. *E. coli* K-12 was grown in BHI medium. At various times, 5-ml samples were collected and assessed for one of the following: (i) number of bacteria (see Fig. 3), (ii) amount of LPS by the LAL assay (see Fig. 3), or (iii) amount of LP by the dot blot assay (Fig. 1). (A) Dot blot assay involving purified LP in BHI medium, was performed at the same time as the dot blot assay in panel B. (B) Dot blot assay of the amount of LP present in culture supernatants obtained at the times indicated. Culture supernatants were assessed undiluted or after being diluted 1:3 or 1:9 in PBS. Data are from one experiment that was representative of five separate experiments.

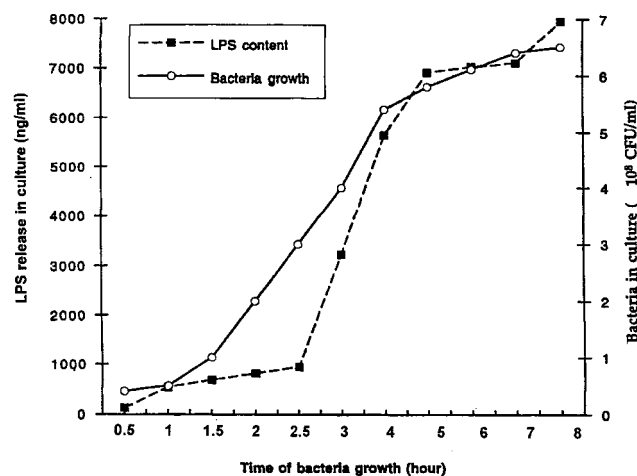


FIG. 3. Bacterial growth and LPS release by *E. coli* K-12. Bacterial growth and LPS contents of bacterial culture supernatants at different times after culture initiation were determined as described in the legend to Fig. 2 and in Materials and Methods. Data are from one experiment that was representative of two separate experiments.

content was determined from a standard curve obtained with *E. coli* LPS. The sensitivity of the assay was 0.1 EU/ml.

Antibiotic treatment of bacteria. *E. coli* cultures were treated with ceftazidime as described by Leeson et al. (29), with minor modifications. Briefly, 2 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) was inoculated with *E. coli* K-12 from an agar plate and incubated for 16 to 18 h at 37°C with orbital shaking at 175 rpm. A 0.2-ml sample of this overnight culture was inoculated into 50 ml of fresh Trypticase soy broth and incubated for 1.5 to 2 h at 37°C with shaking at 175 rpm until bacterial counts reached 3×10^8 to 4×10^8 CFU/ml. Ceftazidime in Trypticase soy broth was added at a final concentration of 20 µg/ml for the first 1.5 h of incubation and then at 600 µg/ml for the last 2.5 h of incubation. The culture was then centrifuged at $10,000 \times g$ for 10 min to remove bacterial debris, and the resulting culture supernatant was assessed for LP content.

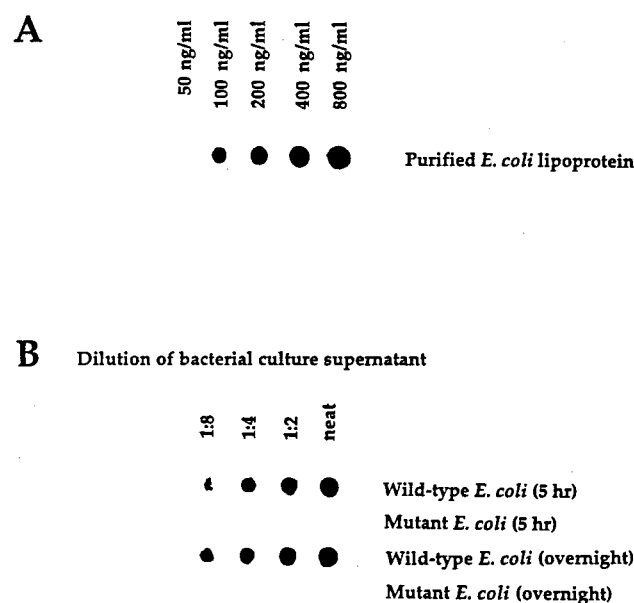


FIG. 4. LP is not detectable in culture supernatants obtained from an *E. coli* LP deletion mutant. *E. coli* K-12 and an *E. coli* mutant (K-12 strain JE5055) that had a deletion of the *lpp* gene were grown, and the culture supernatant was assessed as described in the legend to Fig. 2. Data are from one experiment that was representative of two separate experiments.

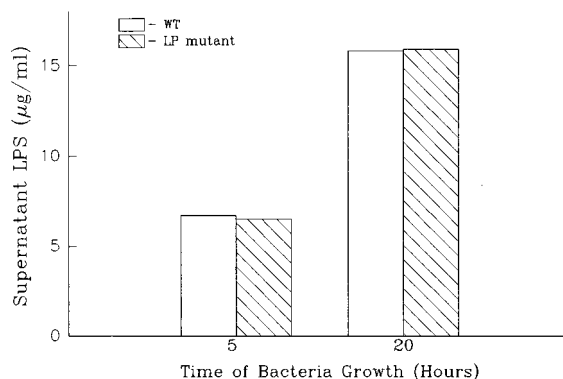


FIG. 5. An *E. coli* LP deletion mutant releases normal levels of LPS during growth. The LPS contents of culture supernatants obtained from wild-type *E. coli* and an *E. coli* LP deletion mutant were assessed as described in the legend to Fig. 3. There was no statistical difference ($P > 0.05$) between the amounts of LPS contained in culture supernatant obtained from wild-type *E. coli* and the LP deletion mutant. Data are from one experiment that was representative of three separate experiments.

In vitro macrophage activation. Peritoneal exudate macrophages were obtained from C3H/HeJ mice as previously described (53). Adherent macrophages were exposed to medium (RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 5% heat-inactivated fetal calf serum) containing various concentrations of bacterial cultural supernatant obtained from *E. coli* K-12 or from the *E. coli* LP deletion mutant (strain 5505). Macrophage culture supernatants were then obtained 8 h after exposure to these *E. coli* culture supernatants and immediately assessed for IL-6 production by enzyme-linked immunosorbent assay, as previously described (53).

RESULTS AND DISCUSSION

In initial experiments, we screened a number of monoclonal antibodies, specific for LP, for their capacity to function in a dot blot assay that could quantitatively detect purified LP present in BHI broth. Using monoclonal antibody 4C4, we developed a dot blot assay that could detect LP at levels as low as 100 ng/ml (Fig. 1). As seen in Fig. 1, LPS was not detected by this assay. Using this dot blot assay, we then assessed *E. coli* culture supernatants obtained at different times after initiation of bacterial growth for the presence of LP. As seen in Fig. 2, LP was present in bacterial culture supernatants and its concentration increased in a time-dependent fashion. LP release was detectable within 30 min of bacterial growth, and substantial levels of LP were released after 5 to 8 h of bacterial growth. This period corresponded to the exponential growth phase of these bacteria, which also was the time when the bacteria released LPS (Fig. 3). Using this dot blot assay, we estimated that 1 µg of LP per ml was released from *E. coli* K-12 after 4 h of culture. While we consider this estimate to be a valid approximation of the amount of LP released by bacteria under these conditions, a more precise determination will be made when a more quantitative assay is developed. This supernatant contained 5.7 µg of LPS per ml as determined by the LAL assay. Thus, LP is present at lower levels (1 to 1.5 µg/ml) than is LPS (5 to 6 µg/ml) in bacterial culture supernatants. However, this concentration of LP is biologically significant when one considers macrophage activation, where 1 ng of LP per ml can activate human or mouse macrophages (51–53). After 4 h of culture, *E. coli* (5.4×10^8) contained 28.6 µg of LPS (53 fg/bacterium) versus 75 µg of LP (148 fg/bacterium). Previous studies have estimated the amount of LPS associated with a single bacterium to be 30 to 40 fg (27, 36, 49). Our dot blot assay was specific for LP, since culture supernatant from an *E. coli* LP deletion mutant had no detectable LP (Fig. 4). How-

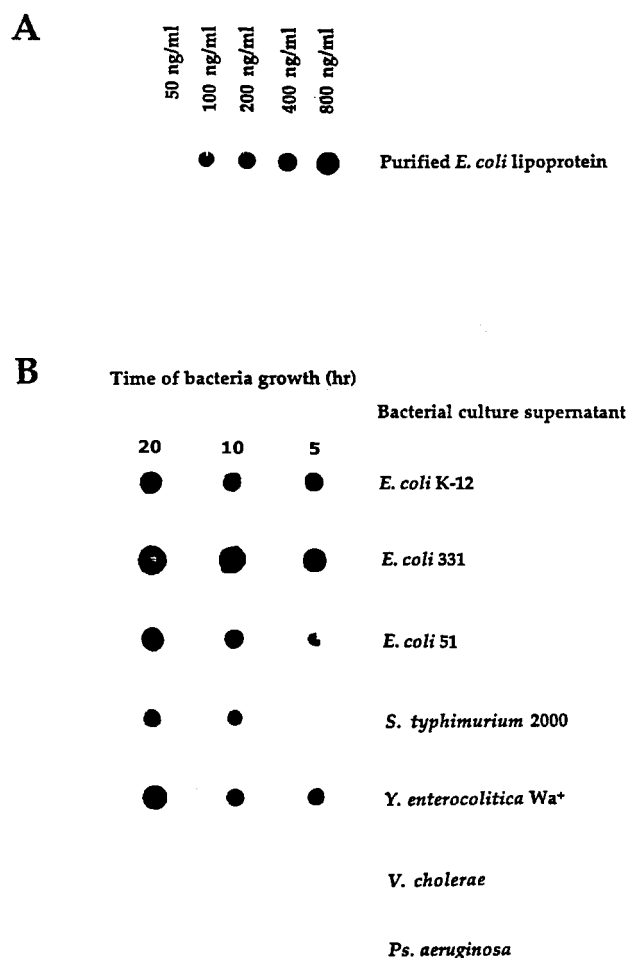


FIG. 6. LP is present in culture supernatants obtained from different bacteria from the family *Enterobacteriaceae*. The LP content in culture supernatants obtained from different bacteria was assessed as described in the legend to Fig. 2. Data are from one experiment that was representative of three separate experiments.

ever, this LP deletion mutant released LPS into the culture medium at levels equivalent to those observed for wild-type *E. coli* (Fig. 5). As seen in Fig. 6, LP release was not unique to *E. coli* K-12. *S. typhimurium* TML, *Y. enterocolitica* WA, and two pathogenic *E. coli* strains (331, an enteropathogenic *E. coli* strain, and 51, an enteroinvasive *E. coli* strain) also released LP into the culture medium during growth. Interestingly, these bacteria varied significantly in the amounts of LP present in their culture supernatants. *E. coli* 331 consistently released the highest LP levels. The significance of these findings is unclear and is currently being investigated. In contrast, no LP could be detected in culture supernatants obtained from bacteria (*V. cholerae* and *P. aeruginosa*) not in the family *Enterobacteriaceae* (Fig. 6).

A number of studies have shown that antibiotic treatment of gram-negative bacteria results in an enhanced release of LPS (2, 15, 16, 29, 32, 42, 47, 48). To investigate whether LP release would also be enhanced following bacterial lysis, we treated *E. coli* with ceftazidime and assessed the culture supernatants for LP content. As seen in Fig. 7, treatment of *E. coli* with this antibiotic resulted in a significant increase in the amount of LP present in bacterial culture supernatants. Compared to control bacterial cultures (no antibiotic treatment), ceftazidime treat-

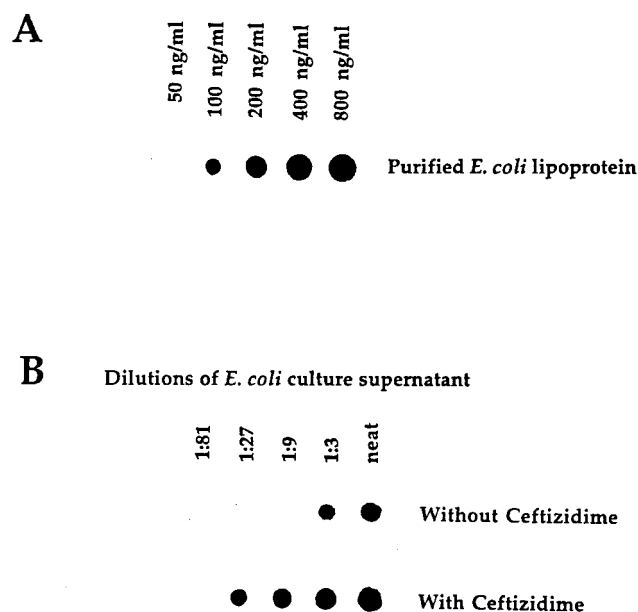


FIG. 7. *E. coli* treated with ceftazidime shows an enhanced release of LP into culture supernatants. Culture supernatants obtained from untreated *E. coli* and *E. coli* treated with ceftazidime were assessed for levels of LP. The culture conditions and ceftazidime treatment are described in Materials and Methods. Data are from one experiment that was representative of three separate experiments.

ment resulted in an approximately ninefold increase in the amount of LP present in the culture supernatant. Thus, the above results indicate that LP is released by growing or lysed bacteria and is present at levels that we have previously shown would activate human and mouse macrophages (51–53). To further explore this issue, we performed experiments that assessed bacteria culture supernatants for their ability to induce cytokine production in mouse macrophages. To minimize the effects of LPS in these experiments, we used C3H/HeJ mice (LPS nonresponsive). Culture supernatants from *E. coli* K-12 and the LP deletion mutant *E. coli* were compared for their ability to induce IL-6 production in peritoneal exudate macrophages. As seen in Fig. 8, *E. coli* K-12 supernatant obtained at 4 h induced significant levels of IL-6 production in macrophages. In contrast, much lower levels of IL-6 were induced in macrophages exposed to equivalent volumes of culture supernatant obtained from the *E. coli* LP deletion mutant (undetectable levels of LP). These results suggest that LP, released by growing or from lysed bacteria, could play an important role in the induction of cytokine production and/or pathology associated with gram-negative bacterial infections. However, the biological significance of LP or LPS release in the complex pathology of gram-negative bacterial infections is currently unclear. This study is the first to show that LP is released by growing or lysed bacteria. This observation, coupled with the fact that LP can activate macrophages, induce lethal shock in mice, and act synergistically with LPS to induce these responses, suggests that LP may play a more important role in bacterial pathogenesis than was previously appreciated. In this regard, we have previously shown that heat-killed preparations of LP-deficient *E. coli* are much less efficient at inducing lethal shock in C3H/HeJ mice than are to heat-killed preparations of wild-type *E. coli* (53).

Our results also indicate that other bacterial components that are also capable of activating macrophages, but different

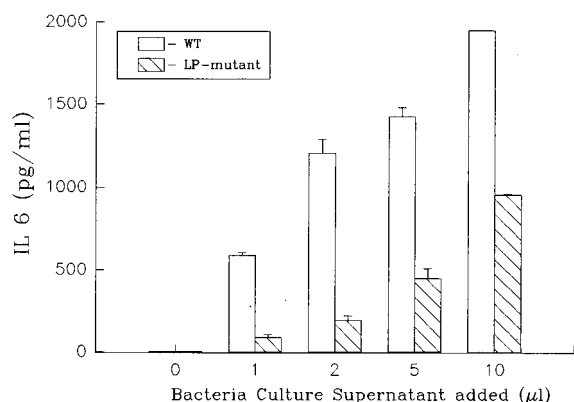


FIG. 8. Supernatants obtained from an *E. coli* LP deletion mutant are less efficient at inducing IL-6 production in macrophages than are supernatants obtained from wild-type *E. coli*. Peritoneal exudate macrophages were obtained from C3H/HeJ mice and exposed to different concentrations of culture supernatant obtained from wild-type *E. coli* and an *E. coli* LP deletion mutant. The supernatants obtained from wild-type and mutant *E. coli* had equivalent levels of LPS (wild-type, 6.4 µg/ml; mutant, 6.1 µg/ml). Macrophage culture supernatants were collected after 8 h of culture and assessed for IL-6 content by enzyme-linked immunosorbent assay as described in Materials and Methods. Levels of IL-6 in supernatant obtained from macrophages stimulated with any of the different concentrations of culture supernatant obtained from the *E. coli* LP deletion mutant were statistically different ($P < 0.01$) from levels of IL-6 in supernatant obtained from macrophages stimulated with culture supernatant obtained from wild-type *E. coli*. Data are from one experiment that was representative of three separate experiments.

from LP or LPS, are released by growing bacteria. Different gram-negative bacteria have been shown to release or produce a number of proteins that could act either alone or in combination with each other or with LP and/or LPS to activate macrophages. In 1945, Binkley et al. (9) reported the isolation of protein from LPS-protein complexes and demonstrated that it was toxic to mice. Since this observation, a number of outer membrane components, different from LPS, have been characterized with regard to their capacity to activate different immune functions. Two such components are endotoxin protein (19, 20, 45) and lipid A-associated protein (7, 35). Endotoxin protein preparations were shown to contain at least 12 proteins, ranging in molecular size from 5 to 80 kDa (19). The predominant proteins present in endotoxin protein preparations were the porins, protein II, and LP (19). It is not known whether all of these proteins are also released by bacteria or synergize with each other to induce cytokine production and/or disease.

In contrast to our results and the above evidence that a number of bacterial proteins can activate monocytes and macrophages, Leeson et al. (29) have published data suggesting that LPS is the predominant proinflammatory mediator in supernatants of antibiotic-treated bacteria. In this study, LPS and proteins contained in bacterial culture supernatants obtained from antibiotic-treated bacteria were separated by isopycnic density gradient ultracentrifugation in cesium chloride or by velocity sedimentation in sucrose gradients. The predominant monocyte-stimulating activity was found in the LPS-containing fractions that were inhibited by polymyxin B. Surprisingly, the protein fractions obtained by these purification techniques had little to no activity with regard to activating monocytes. Since we have previously shown (51–53) that LP is as potent as LPS at activating human and mouse macrophages, LP and possibly other proteins may have been lost or altered as a result of the purification techniques used in this study. Alternatively, LP

and/or other proteins may have aggregated and/or formed complexes with LPS.

LP is the most abundant protein in the outer membrane of bacteria in the *Enterobacteriaceae* (16, 45). Melchers et al. (33) were the first to show that LP was mitogenic for mouse B cells. Much of the current knowledge of the biologic properties of LP has come from work by Bessler and colleagues (4–6, 8, 21, 24). LP and synthetic lipopeptides activate mouse and human macrophages (21, 24). LPs from bacteria not in the *Enterobacteriaceae* could also play important roles in the pathologic findings in diseases caused by these bacteria. For example, spirochetal LPs are key inflammatory mediators in syphilis and Lyme disease. LPs obtained from *Treponema pallidum* and *Borrelia burgdorferi* or lipopeptides corresponding to the N-terminal domains of these LPs activate monocytes/macrophages, B cells, and endothelial cells (12, 31, 41, 46, 50). Interestingly, these LPs were shown to activate cells via signaling pathways and/or receptors that appear to be different from those used by LPS (37, 39, 40). The possibility that these LPs are also released during growth and/or lysis has not been fully explored.

In summary, we have shown that LP is released by growing bacteria and that this release is dramatically enhanced after treatment with the antibiotic ceftazidime. More importantly, LP is released at levels that can induce cytokine production in macrophages. Collectively, these results suggest that LP plays an important role in the pathology associated with infections caused by gram-negative bacteria from the family *Enterobacteriaceae*.

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